THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 7, pp. 4326–4335, February 16, 2007
Printed in the U.S.A.

Functional Characterization of Desaturases Involved in the Formation of the Terminal Double Bond of an Unusual 16:3$\Delta^{9,12,15}$ Fatty Acid Isolated from *Sorghum bicolor* Root Hairs*

Received for publication, July 3, 2006, and in revised form, December 18, 2006 Published, JBC Papers in Press, December 18, 2006, DOI 10.1074/jbc.M606343200

Zhiqiang Pan[‡1,2], **Agnes M. Rimando**[‡1], **Scott R. Baerson**[‡], **Mark Fishbein**[§], **and Stephen O. Duke**[‡]

From the [‡]*United States Department of Agriculture, Agricultural Research Service, Natural Products Utilization Research Unit, University of Mississippi, University, Mississippi 38677 and the* [§]*Department of Biology, Portland State University, Portland, Oregon 97207*

Sorgoleone, produced in root hair cells of sorghum (*Sorghum bicolor*), is likely responsible for much of the allelopathic properties of sorghum root exudates against broadleaf and grass weeds. Previous studies suggest that the biosynthetic pathway of this compound initiates with the synthesis of an unusual 16:3 fatty acid possessing a terminal double bond. The corresponding fatty acyl-CoA serves as a starter unit for polyketide synthases, resulting in the formation of 5-pentadecatrienyl resorcinol. This resorcinolic intermediate is then methylated by an *S*-adenosylmethionine-dependent *O*-methyltransferase and subsequently dihydroxylated, yielding the reduced (hydroquinone) form of sorgoleone. To characterize the corresponding enzymes responsible for the biosynthesis of the 16:3 fatty acyl-CoA precursor, we identified and cloned three putative fatty acid desaturases, designated *SbDES1*, *SbDES2*, and *SbDES3*, from an expressed sequence tag (EST) data base prepared from isolated root hairs. Quantitative real-time RT-PCR analyses revealed that these three genes were preferentially expressed in sorghum root hairs where the 16:2 and 16:3 fatty acids were exclusively localized. Heterologous expression of the cDNAs in *Saccharomyces cerevisiae* revealed that recombinant SbDES2 converted palmitoleic acid (16:1Δ^9) to hexadecadienoic acid (16:2$\Delta^{9,12}$), and that recombinant SbDES3 was capable of converting hexadecadienoic acid into hexadecatrienoic acid (16:3$\Delta^{9,12,15}$). Unlike other desaturases reported to date, the double bond introduced by SbDES3 occurred between carbons 15 and 16 resulting in a terminal double bond aliphatic chain. Collectively, the present results strongly suggest that these fatty acid desaturases represent key enzymes involved in the biosynthesis of the allelochemical sorgoleone.

Numerous plant species produce phytotoxic secondary metabolites, some of which may play a direct role in allelopathic interactions (1, 2). These interactions often represent a form of chemical warfare occurring between neighboring plants competing for limited light, water, and nutrient resources (2, 3). Several *Sorghum* species have been reported to produce phytotoxins, which are exuded from their root systems into the rhizosphere, which suppress the growth of competing species (4). Numerous studies have contributed to the discovery and identification of the chemicals that are responsible for this observed allelopathic inhibition. For example, studies on the biologically active components of both water-soluble and water-insoluble exudates from roots of *Sorghum bicolor* have demonstrated their role in the growth inhibition of lettuce seedlings (*Lactuca sativa*), as well as a number of important invasive weed species (5). The major constituent of these exudates was identified as 2-hydroxy-5-methoxy-3-[(8′*Z*,11′*Z*)-8′,11′,14′-pentadecatriene]-*p*-benzoquinone, referred to as sorgoleone (Fig. 1) (6). Early reports on the phytotoxicity of sorgoleone indicated that it is a strong inhibitor of CO_2-dependent oxygen evolution in plastids (7). Further studies on its mode of action have documented additional effects on both photosynthetic and mitochondrial electron transport (8–11).

The herbicidal and allelopathic properties of sorgoleone make the isolation and characterization of the corresponding genes involved in sorgoleone biosynthesis highly desirable, as manipulation of the pathway in sorghum, or genetic modification of other plant species using these genes could provide important insights into the underlying allelochemical interactions involved (12). Sorgoleone biosynthesis is likely exclusive to root hairs, which appear as cytoplasmically dense cells in sorghum, containing large osmiophilic globules deposited between the plasma lemma and cell wall, presumably associated with sorgoleone rhizosecretion (13, 14). Labeling studies have demonstrated that the biosynthesis of sorgoleone involves the convergence of the fatty acid and polyketide pathways (15, 16), likely through the action of a novel type III polyketide synthase activity utilizing fatty acyl-CoA starter units, resulting in the addition of a quinone head via iterative condensation of acetate extender units. Subsequent modifications of the alkylresorcinol intermediate are mediated by *S*-adenosylmethionine-dependent *O*-methyltransferases and possibly P450 monooxygenases, yielding the reduced form of sorgoleone (a hydroquinone). Upon exudation, the less stable hydroquinone rapidly oxidizes to the highly active benzoquinone form, which can persist in

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) EF206346, EF206347, and EF206348.

[1] These authors contributed equally to this work.

[2] To whom correspondence should be addressed: USDA-ARS Natural Products Utilization Research Unit, P. O. Box 8048, University, MS 38677. Tel.: 662-915-1548; Fax: 662-915-1035; E-mail: zpan@msa-oxford.ars.usda.gov.

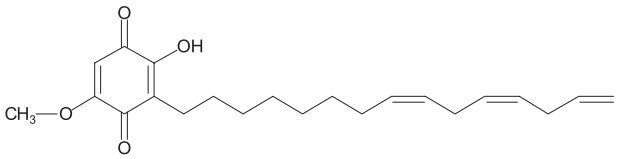

FIGURE 1. **Chemical structure for sorgoleone.**

soil for extended periods (11, 17, 18). A highly unusual characteristic of the sorgoleone molecule is the presence of a terminal double bond in the aliphatic side chain derived from a desaturated 16:3 fatty acyl-CoA precursor. Although the biochemical pathways involved in biosyntheses of many commonly occurring polyunsaturated fatty acids (PUFAs) in plants have been well characterized (19–21), to our knowledge, enzymes capable of performing desaturation reactions producing a terminal double bond have not previously been characterized in any system.

In an attempt to isolate genes encoding fatty acid desaturases involved in the sorgoleone biosynthetic pathway, we have utilized an expressed sequence tag (EST)[3] data base containing ~5,500 sequences randomly selected from a cDNA library prepared from purified *S. bicolor* (genotype BT×623) root hairs.[4] In this report, we describe the cloning and functional characterization of two fatty acid desaturases, designated SbDES2 and SbDES3, which consecutively convert 16:1 fatty acid to 16:3 fatty acid having a terminal double bond, identified from mining of the sorghum root hair EST data base.[5] Yeast cells overexpressing the *SbDES3* cDNA were able to convert 16:3$\Delta^{9,12}$ fatty acid substrate to its 16:3$\Delta^{9,12,15}$ trienoic form, which is considered to be the substrate used by polyketide synthases in the sorgoleone biosynthetic pathway (15, 16). To our knowledge, this is the first plant fatty acid desaturase described to date exhibiting terminal desaturase activity. Furthermore, the tissue-specific accumulation of *SbDES2* and *SbDES3* transcripts correlates with the accumulation of 16:2 and 16:3 fatty acids in root hairs (as determined by GC/MS), strongly suggesting the participation of these enzymes in the sorgoleone biosynthetic pathway.

EXPERIMENTAL PROCEDURES

Plant Material—Mature leaves, stems, and emerging panicles were harvested from ~2-month-old, greenhouse-grown *Sorghum bicolor* (cv. BT×623) plants. Immature leaves and shoot apices were isolated from 8-day-old BT×623 seedlings maintained in a growth chamber at 28 °C, 16 h light/8 h dark, 400 μmol/m^{2}sec intensity. Total root systems and root hairs were isolated from 8-day-old seedlings grown using a capillary mat system (14). All tissues were collected, then flash-frozen in liquid nitrogen, and kept at −80 °C prior to extraction. Root hairs were isolated according to the method of Bucher *et al.* (22).

RNA Isolation and Quantitive Real-Time RT-PCR—Total RNAs for real-time PCR experiments shown in Fig. 3 were isolated from 50 mg of flash-frozen, pulverized 10-day-old BT×623 seedling tissues using an RNeasy Plant Mini-Kit (Qiagen, Valencia, CA). Quantitative real-time RT-PCR (QRT-RTPCR) reactions were performed in triplicate using a GenAmp® 5700 Sequence Detection System (Applied Biosystems, Foster City, CA) as previously described (23).

Isolation of Sorghum cDNA Clones—To obtain full-length cDNA clones, both 5′- and 3′ rapid amplification of cDNA ends (RACE) was carried out using the BD SMART™ RACE cDNA Amplification Kit (BD Biosciences Clontech, Palo Alto, CA) according to the manufacturer's instructions. Primer sets for 5′- and 3′-RACE were 2A03: 5′-CCAAGGAGGTGAAGTGGCAG-3′ and 5′-ATACTACCGGGAGCCACACAAG-3′; 25B05: 5′-TCGCTGACGAAATGGTTGAC-3′ and 5′-CCTCCTTGGCGTGTTCCTC-3′; 56D10: 5′-TGGACGATCACCTCAATCCTG-3′ and 5′-CAACAAGTTCTAGCTGCTTGATGC-3′, respectively. Products of the RACE amplifications were resolved on agarose gels, cloned into the pCR®4-TOPO vector (Invitrogen, Carlsbad, CA), and then confirmed by sequence analysis. Full-length cDNAs were then amplified with primer pairs complementary to the 5′- and 3′-ends of ORFs identified in RACE experiments, using *Pfu* thermostable DNA polymerase (Stratagene, La Jolla, CA) and first-strand cDNA generated from RNA extracted from sorghum root hairs. Several independent isolates from each amplification were sequenced to ensure the authenticity of the ORFs.

Phylogenetic Analysis—Amino acid sequences of putative homology to fatty acid desaturases were retrieved from GenBank™ using BLAST with standard default parameters. A data set was assembled from 54 sequences, in addition to the three sequences characterized here (SbDES 1–3). Sequences of the chlorophyte, *Chlorella vulgaris*, were included to root the phylogeny. Multiple sequence alignments were constructed with ClustalX ver. 1.81 (24). Three parameter sets were investigated to assess sensitivity to gap costs: default (gap opening, 10.0; gap extension, 0.2), (10.0, 1.0), and (1.0, 1.0). The alignments differed somewhat in length (463, 464, and 482 residues, respectively). Phylogenetic estimates were conducted separately for each alignment. The neighbor-joining method (25) as implemented in PAUP* ver. 4.0b10[6] was used with default parameters, except that ties were broken randomly. Trees were midpoint rooted and nodal support was estimated by the bootstrap (27), employing 5000 pseudoreplicate data sets. Trees estimated from the three alignments were extremely similar. The second alignment, with a 10:1 ratio of gap opening to extension penalties, was selected for further analysis. However, all interpretations made here would be identical on trees estimated from the other two alignments (not shown).

[3] The abbreviations used are: EST, expressed sequence tag; GC, gas chromatograph; MS, mass spectrometry; FAME, fatty acid methyl esters; FAD, fatty acid desaturase; NMR, nuclear magnetic resonance; RACE, rapid amplification of cDNA ends; DMOX, 4,4-dimethyloxazoline; ORF, open reading frame.

[4] S. R. Baerson, F. E. Franck E. Dayan, A. R. Agnes M. Rimando, N. P. D. Nanayakkara, C. Liu, J. Schröder, M. Fishbein, Z. Pan, I. A. Kagan, L. H. Pratt, M. Cordonnier-Pratt, and S. O. Duke, submitted manuscript.

[5] Fatty acid nomenclature: X:Y indicates that the fatty acid contains X number of carbon atoms and Y number of double bonds. Δ^Z indicates that a double bond is located at *z*th carbon atom counting from the carboxyl group. No designation for the configuration of double bond is used when the double bond in the fatty acid is in *cis* configuration.

[6] D. Swofford (2001) PAUP* Phylogenetic Analysis Using Parsimony (*, other methods), Version 4, Sinauer Associates, Sunderland, MA.

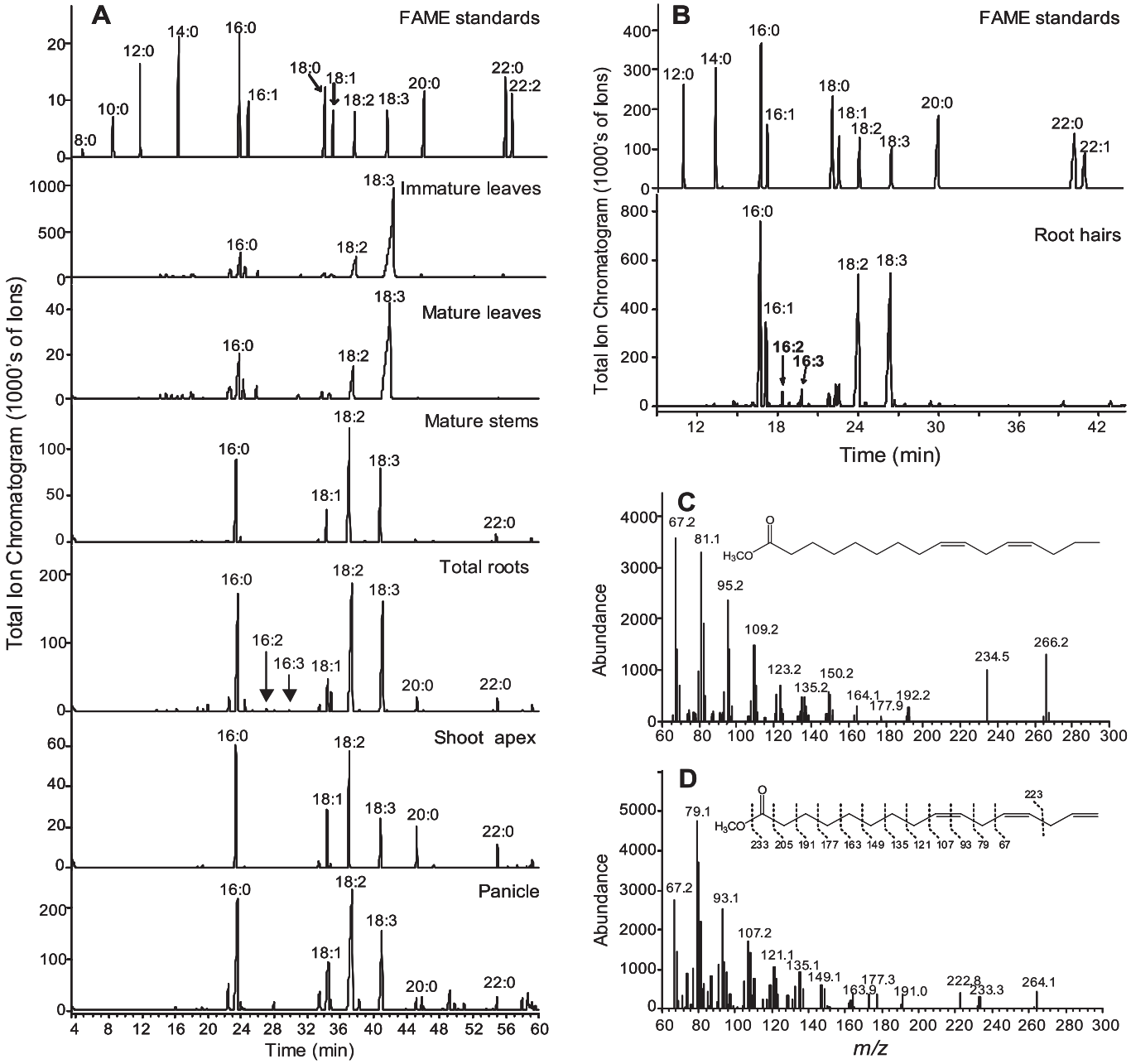

FIGURE 2. **Fatty acid profiles of different sorghum tissues.** *A*, gas chromatograms of FAMEs prepared from immature leaves, mature leaves, mature stems, total root systems, shoot apices, and panicles; *B*, gas chromatogram of FAMEs prepared from root hair cells, showing the presence of 16:2 and 16:3 fatty acids (FAMEs of authentic standards shown above); *C*, corresponding mass spectrum for 16:2$\Delta^{9,12}$ fatty acid identified in root hairs; *D*, corresponding mass spectrum for 16:3$\Delta^{9,12,15}$ fatty acid identified in root hairs.

Plasmid Construction for Heterologous Expression in S. cerevisiae—For heterologous expression in yeast, ORFs were cloned as HindIII/XhoI fragments 3′ to the galactose-inducible GAL1 promoter in pYES2 (Invitrogen), yielding the plasmids pYE25B (for SbDES1 overexpression), pYE56D (for SbDES2 overexpression), and pYE2A (for SbDES3 overexpression). All three constructs were then transformed into yeast strain INVSc1 using the lithium acetate method (28).

To construct additional vectors for co-expression of two ORFs in yeast, the galactose-inducible promoter::*SbDES3* (or *SbDES1*)::terminator expression cassettes were subcloned into the yeast shuttle vector pRS423 containing a his+ marker (29). To achieve this, expression cassettes were amplified from pYE2A and pYE25B using the PCR primers Lgal (5′-gggcgcgccACGGATTAGAAGCCGCCGAG-3′) and Rcyc (5′-gggcgcGCCGCAAATTAAAGCCTTCGA-3′), cloned into the pCR®4-TOPO vector (Invitrogen) and confirmed by DNA sequence analyses. The expression cassettes were then excised using BssHII (for *SbDES3*) or AscI (for *SbDES1*), gel-purified, and subcloned into BssHII or AscI-digested pRS423, yielding pRS2A (containing *SbDES3*) and pRS25B (containing *SbDES1*). These plasmids were then transformed into the yeast strain INVSc1 harboring pYE56D as described above. All yeast transformants were confirmed by colony-PCR using gene specific primers, and by further restriction analyses performed using isolated plasmid preparations.

Culture Conditions and Recombinant Protein Expression—Transformed yeast cells were grown in synthetic medium (SC-Ura) containing 2% (w/v) glucose as carbon source and 0.67% (w/v) yeast nitrogen base without amino acids. In the case of co-expression, transformed yeast cells were grown in similar media lacking histidine (SC-UH) for the selection of pRS423. Induction of recombinant protein expression was performed as described by Dyer *et al.* (30): briefly, overnight cultures were pelleted by centrifugation, and resuspended in induction medium (2% (w/v) galactose, 0.67% (w/v) yeast nitrogen base without amino acids, and 0.1% (v/v) tergitol Nonidet P-40 (Sigma). The cells then were diluted in 20 ml of same medium to obtain an A_{600} of 0.4. Exogenous free fatty acids (Sigma), when included, were added to the diluted cells at a final concentration of 0.1% (v/v). Yeast cell lines, including empty vector controls, were induced at 20 °C for 48 h prior to harvest, both in the absence or presence of exogenous fatty acids added to cultures at a final concentration of 0.1% (v/v): palmitic acid, palmitoleic acid, oleic acid, linoleic acid, γ-linolenic acid, and α-linolenic acid (Sigma). For *SbDES2* and *SbDES3* co-expression experiments, yeast cultures were maintained at 30 °C to obtain optimal yields of the 16:3$\Delta^{9,12,15}$ product (Fig. 6, *C* and *F*; Table 1).

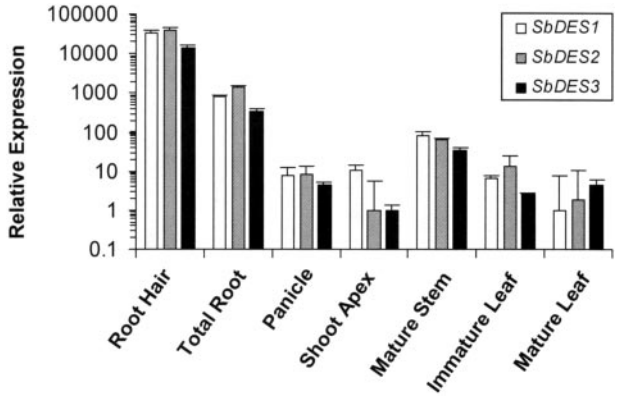

FIGURE 3. **Expression of different FAD-like ESTs in sorghum.** Relative expression levels of 3 FAD-like sequences were determined in different sorghum tissues by quantitative real-time RT-PCR, using gene-specific primers. Data were normalized to an internal control (18 S rRNA), and the $\Delta\Delta C_T$ method was used to obtain the relative expression levels for each sequence. Data are expressed as mean ± S.D.

Fatty Acid Analyses—Cellular fatty acids from yeast and plant tissues were extracted according to the method of Peyou-Ndi *et al.* (31). Fatty acids were analyzed as methyl ester derivatives on an Agilent 6980GC (Agilent Technologies, Foster City, CA) coupled to a JEOL GCMate II mass spectrometer (JEOL USA, Inc., Peabody, MA) using an Omegawax 250™ (Supelco, St. Louis, MO) capillary column, 30 m × 0.25 mm × 0.25 μm film thickness. The fatty acids were identified by comparison of their retention times and mass spectra with those of authentic standards (Sigma). The inlet (splitless), GC interface, and ion chamber temperatures were 250, 250, and 230 °C, respectively. The volume of sample injected was 1 μl. The GC temperature program for analysis of the hexane extracts, with the exception of the root hair sample, was initial 110 °C held for 5 min, raised to 160 °C at a rate of 10 °C/min, raised to 190 °C at the rate of 1 °C/min, raised to 280 °C at the rate of 2 °C/min and held at this temperature for 2 min. The carrier gas was ultra high purity helium, flow rate 1 ml/min. The root hair hexane extract was analyzed using the following temperature program: initial 80 °C held for 3 min, raised to 170 °C at the rate of 10 °C/

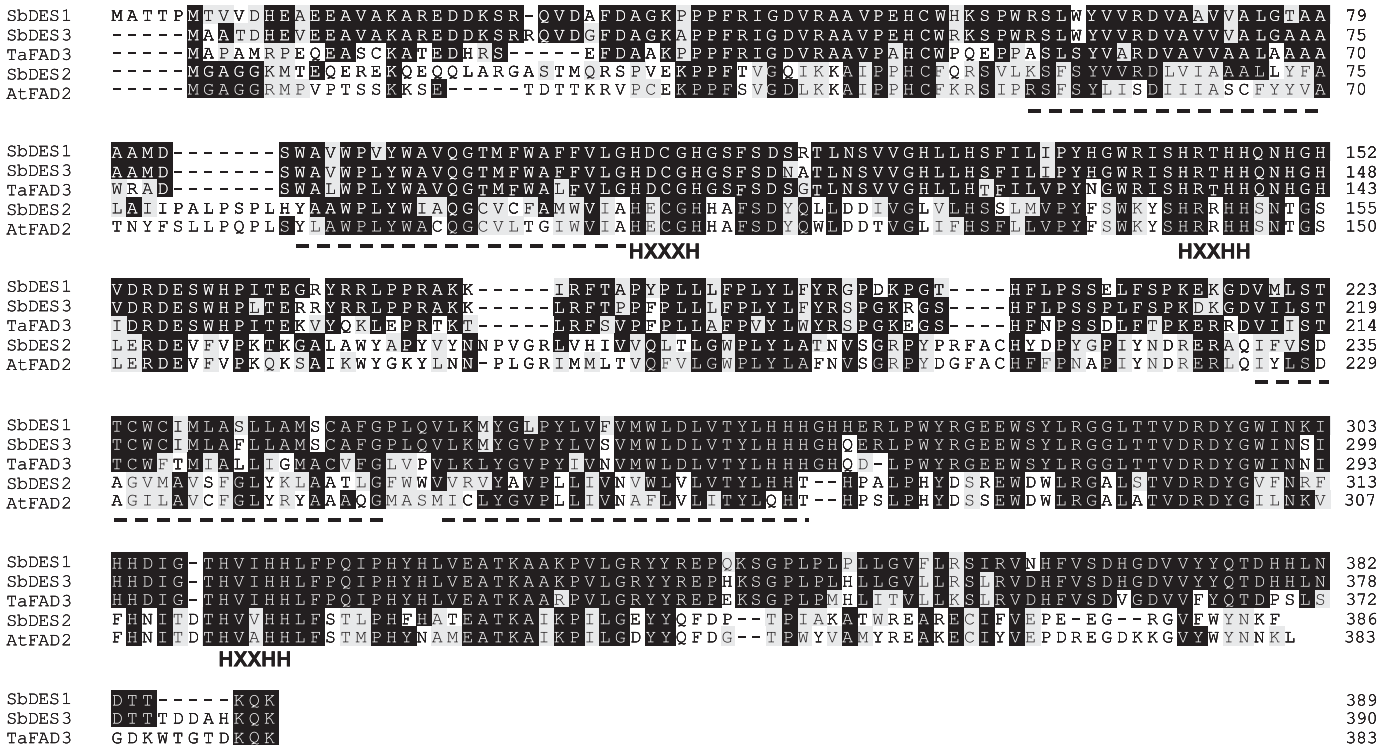

FIGURE 4. **Sequence alignment of SbDES1, 2, and 3 with related plant FADs.** Amino acid sequences were aligned using ClustalW. The three conserved histidine motifs are indicated, and the predicted transmembrane domains denoted by *dashed lines*. Identical and similar amino acids are shaded with *black* and *gray*, respectively. Abbreviations: AtFAD2, *Arabidopsis thaliana* FAD2 (GenBank™ accession number: AAM98321); TaFAD3, *Triticum aestivum* (GenBank™ accession number: BAA28358).

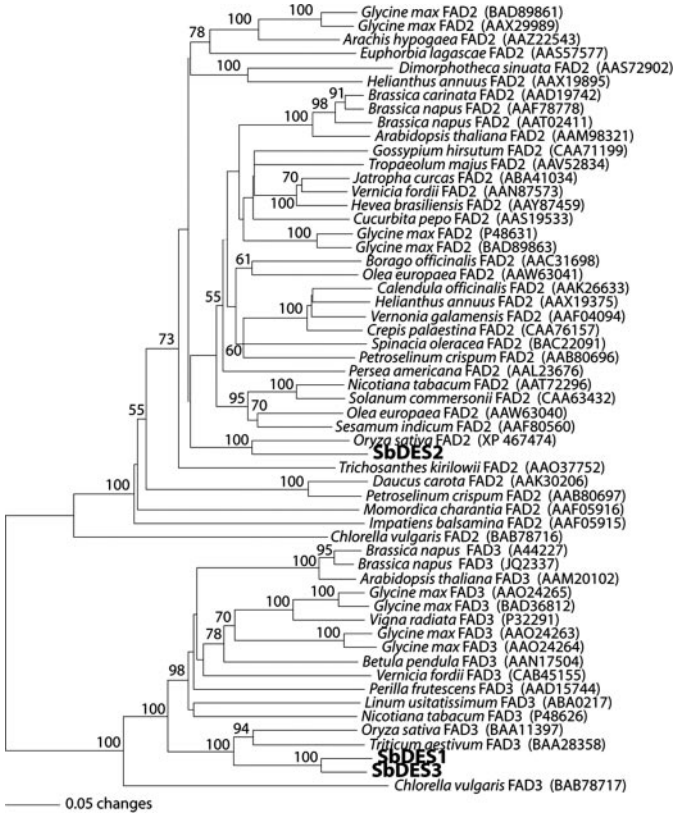

FIGURE 5. **Phylogenetic analysis of SbDES1, SbDES2, and SbDES3 relatives.** The phylogram was generated using the neighbor-joining method as implemented in PAUP ver 4.0b10. The bootstrap method was performed for 5000 pseudoreplicate data sets. Numbers shown at the tree forks indicate frequency of occurrence among all bootstrap iterations performed.

min, raised to 200 °C at the rate of 1 °C/min, raised to 280 °C at the rate of 5 °C/min and held at this temperature for 2 min. For further confirmation of double bond positions, methyl esters were converted into their 4,4-dimethyloxazoline (DMOX) derivatives as previously described (32), except that the derivatization reactions were performed at 170 °C for 15 h. DMOX derivatives were analyzed by GC/MS using the same conditions as described above for the analysis of fatty acid methyl ester derivatives.

Isolation and Analysis of 9,12,15(9Z,12Z)-Hexadecatrienoic Acid (16:3 FA) by GC/MS and Nuclear Magnetic Resonance (NMR) Spectroscopy—The 16:3 fatty acid generated by yeast cells overexpressing the *SbDES2* and *SbDES3* cDNAs was extracted with hexane and isolated by thin layer chromatography using aluminum-backed silica F_{254} plates (10×20 cm^2, 0.2 mm silica thickness; EM Science) impregnated with $AgNO_3$. The silica gel plates were pretreated with a solution of 5% $AgNO_3$ in MeOH-H_2O (95:5, v/v), air-dried for 60 min, placed in an oven at 80 °C for 16 h, and allowed to cool to room temperature prior to use. Extracts were then applied to plates and developed using CH_2Cl_2:ethyl acetate (88:12). The 16:3 fatty acid migrated as a band of R_f 0.12, which was scraped from the plate, extracted with $CHCl_3$, and then dried under a stream of nitrogen. An aliquot was analyzed by GC/MS using the same conditions as those described above for the yeast and plant tissue extracts. The 16:3 fatty acid was further characterized by ^{1}H NMR spectroscopy (Bruker DRX 400 UltraShield™ spectrometer, XWIN-NMR Software Version 3.1; Bruker, Billerica, MA). ^{1}H NMR ($CDCl_3$): δ 5.80 (m, 1H, H-15); 5.30 (m, 4H, H-9, 10, 12, 13); 4.98 (br d, $J = 17$ Hz, 1H, H-16a); 5.02 (br d, $J = 10$ Hz, 1H, H-16b); 4.90–5.01 (br dd, 2H, H-16); 3.71 (s, 3H, OCH_3), 2.78 (m, 4H, H-11, 14), 2.32–2.36 (m, 2H, H-2), 2.00 (m, 2H, H-8), 1.49 (m, 2H, H-3), 1.25–1.29 (m, 8H, H-4, 5, 6, 7).

RESULTS

Fatty Acid Composition Analyses in Sorghum Tissues—The biosynthesis of the allelochemical sorgoleone is a multistep process involving both plastidic and cytoplasmic enzymes (15, 16). One of the key steps is the formation of hexadecatrienoic acid (16:3$\Delta^{9,12,15}$), an unsaturated fatty acid proposed to serve as a precursor for the associated PKS in the pathway. To examine the presence and distribution of 16:2$\Delta^{9,12}$ and 16:3$\Delta^{9,12,15}$ in *S. bicolor*, the fatty acid compositions of various tissues (mature and immature leaves, stems, shoot apices, panicles, and whole root systems) were analyzed (Fig. 2*A*). The predominance of palmitic (16:0), linoleic (18:2), and linolenic (18:3) acids was observed in all sorghum tissues. Traces of 16:2 (peak at 27.0 min) and 16:3 (peak at 29.7 min) were observed in the chromatograms of extracted roots, as indicated by mass spectral data (not shown); however the low levels for these peaks precluded their unequivocal identification. As sorgoleone is a major component in the oily exudate droplets associated with sorghum root hairs (13, 14), root hairs were also isolated and analyzed for the presence of the precursor C_{16} fatty acids. Two peaks (corresponding to 16:2 and 16:3 fatty acid) were detected in the lipid extracts (Fig. 2*B*) by GC/MS. The mass spectrum (Fig. 2*C*) of the fatty acid methyl ester (FAME) corresponding to the 16:2 peak (Fig. 2*B*) exhibited a prominent molecular ion at $m/z = 266.2$, characteristic of a 16:2 methyl ester and identical to that of methyl 9,12-hexadecadienoate, whereas the spectrum of the second peak corresponding to 16:3 (Fig. 2*B*) had a molecular ion at $m/z = 264.1$, and a fragmentation pattern consistent with hexadecatrienoate possessing a terminal methylene (Fig. 2*D*). Collectively, these data identify the 16:3$\Delta^{9,12}$ and 16:3$\Delta^{9,12,15}$ fatty acids in root hairs, and furthermore, strongly suggest that these precursors are primarily synthesized in root hair cells.

Identification and Cloning of Fatty Acid Desaturases from Sorghum—To identify the desaturases associated with the sorgoleone biosythetic pathway, particularly those involved in the terminal bond desaturation of C_{16} fatty acid, an EST data base generated using a cDNA library prepared from isolated sorghum root hair cells was mined. Data base mining was performed both by using the Magic Gene Discovery software (33) and by BLAST analysis. From these analyses, we identified 47 desaturase-like ESTs, which assembled into 11 unique contigs. Quantitative real-time RT-PCR analysis showed that 3 of 11 unique desaturase sequences (suggested by clustering) were preferentially expressed in root hair cells (Fig. 3). The corresponding full-length cDNA clones were isolated using cDNA prepared from root hair cells, and designated *SbDES1*, *SbDES2*, and *SbDES3*. The protein sequences deduced from *SbDES3* and *SbDES1* were 90% identical to each other, and shared only 33.7% and 33.8% identity, respectively, with the sequence from *SbDES2*. BLAST analysis of these protein sequences revealed

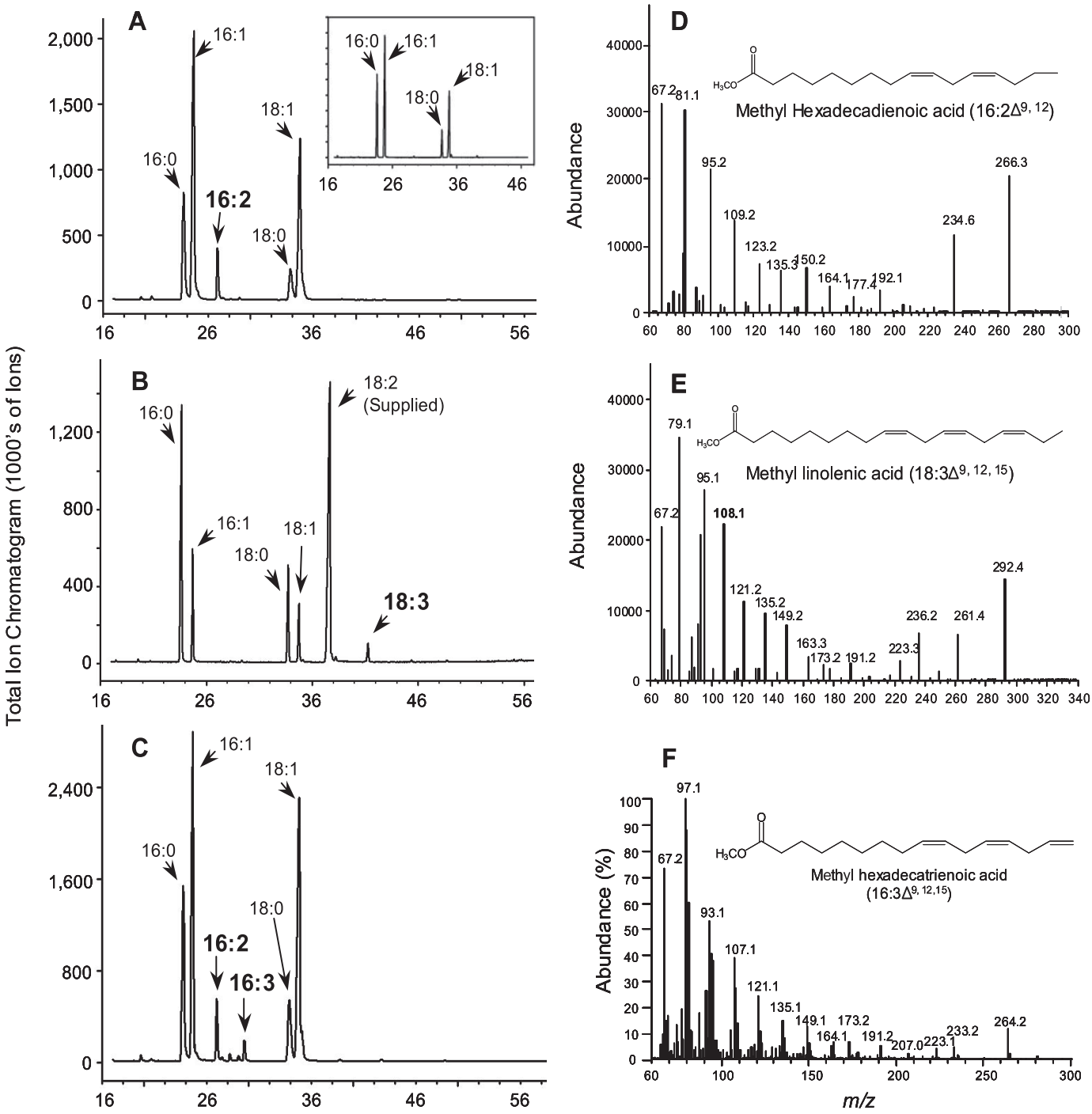

FIGURE 6. **GC/MS analyses of FAMEs prepared from yeast cells expressing *S. bicolor* desaturases, cultured in the presence and absence of exogenously supplied fatty acids.** *A–C*, gas chromatograms of FAMEs: (*A*), cells expressing *SbDES2*, cultured without exogenous fatty acids (*inset*: FAMEs prepared from yeast transformed with empty vector pYES2); *B*, cells expressing *SbDES3*, cultured with exogenous linoleic acid (18:2$\Delta^{9,12}$); *C*, cells co-expressing *SbDES2* and *SbDES3*, cultured without exogenous fatty acids. *D–F*, mass spectra of corresponding fatty acids produced in transformed yeast cells: *D*, 16:2 fatty acid from *SbDES2*-expressing cells; *E*, 18:3 fatty acid from *SbDES3*-expressing cells; *F*, 16:3 fatty acid from cells co-expressing *SbDES2* and *SbEDS3*.

that both SbDES3 and SbDES1 exhibited significant similarity to known plant fatty acid desaturase (FAD3-type) sequences, and SbDES2 displayed a high degree of similarity to plant FAD2-type sequences (34, 35). A previously isolated putative desaturase clone from the *S. bicolor* × *S. sudanense* hybrid, SX17 (36), showed homology to the *SbDES3* clone described in this article. Based on comparisons to known FADs, the predicted protein sequences of all three cDNAs exhibited all of the major structural features possessed by FADs from other systems (34, 35), notably the predicted transmembrane domains and three highly conserved histidine-rich motifs occurring in most membrane-bound desaturases (Fig. 4). The conserved histidine-rich motifs are believed to be required for fatty acid desaturase activity (37).

These comparisons were further supported by phylogenetic analyses (Fig. 5). SbDES2 was most closely related to members

TABLE 1

Accumulation of fatty acid products in transformed yeast

Products were identified by GC/MS as fatty acid methyl ester derivatives (see "Experimental Procedures"). For transgenic yeast harboring pYE2A (*SbDES3*), exogenous linoleic acid (18:2$\Delta^{9,12}$) was supplemented in the culture medium. Values are expressed as the mean percentage among detectable C_{12}–C_{18} fatty acids.

Gene	Products accumulated		
	C16:2	C16:3	C18:3
SbDES2	4.4 (4.2, 4.5)	ND[a]	ND[a]
SbDES3	ND[a]	ND[a]	2.1 (2.3, 1.9)
SbDES2/3	4.3 ± 0.3	1.6 ± 0.2	ND[a]

[a] ND, not detected.

of the plant microsomal FAD2-type desaturase subfamily which, among those functionally characterized, typically desaturate C_{16}/C_{18} acyl chains at the Δ^{12} position. In contrast, both SbDES1 and SbDES3 were positioned within the group corresponding to the plant microsomal FAD3-type desaturases, which mainly catalyze the conversion of 18:2 to 18:3 in phospholipids (38). For example, the closely related FAD3-like enzymes from wheat and rice (GenBank™ accession nos. BAA28358 and BAA11397 - Fig. 5) catalyze the conversion of linoleic acid (18:2$\Delta^{9,12}$) to linolenic acid (18:3$\Delta^{9,12,15}$) (39, 40).

Functional Characterization of FAD-like cDNAs in S. cerevisiae—For functional characterization of the putative FADs, vectors were engineered for heterologous expression in S. cerevisiae. The complete ORFs for SbDES1, SbDES2, and SbDES3, were cloned into the yeast expression vector pYES2, resulting in the vectors pYE25B, pYE56D, and pYE2A, respectively. Constructs were transformed into the *S. cerevisiae* strain INVSc1, and the resulting transformants were cultivated in the presence and absence of exogenously supplied palmitic (16:0), palmitoleic (16:1Δ^9), oleic (18:1Δ^9), linoleic (18:2$\Delta^{9,12}$), α-linolenic (18:3$\Delta^{9,12,15}$), or γ-linolenic acid (18:3$\Delta^{6,9,12}$). As a control, strains harboring the empty pYES2 vector were cultured in parallel. Following 48 h of incubation, the cells were harvested, lipids were extracted, and fatty acid compositions were determined by GC/MS analysis of fatty acid methyl ester derivatives.

As expected, the fatty acid composition of yeast cells transformed with the empty vector (pYES2) alone revealed a simple profile consisting of the four predominant fatty acids (16:0, 16:1Δ^9, 18:0, and 18:1Δ^9) typically found in wild-type yeast (Ref. 41; Fig. 6*A*, *inset*). Expression of *SbDES1* in yeast cells yielded no additional peaks, regardless of whether or not fatty acids were exogenously provided (not shown). In contrast, expression of *SbDES2* resulted in the appearance of one additional peak with a retention time of 27.26 min under all culture conditions, which accounted for ~4.4% of the total cellular fatty acids when exogenous fatty acids were not provided (Fig. 6*A* and Table 1). This peak was identified as 16:2$\Delta^{9,12}$ by mass spectral analysis (Fig. 6*D*), suggesting that endogenous palmitoleic acid (16:1Δ^9) served as substrate for SbDES2. The fragmentation pattern of the mass spectrum obtained (Fig. 6*D*) was identical to one previously published for 16:2$\Delta^{9,12}$, and was further confirmed by GC/MS analysis of 2,4-dimethyloxazoline (DMOX) derivatives (Fig. 7*A*). SbDES2 exhibited no detectable Δ^{12} linoleate desaturase activity, despite the presence of significant endogenous amounts of oleic acid (Fig. 6*A*, *inset*). Of particular significance was the observation that the mass spectrum of the 16:2$\Delta^{9,12}$ FAME (Fig. 6*D*) was also identical to that of the 16:2 fatty acid extracted from sorghum root hairs (Fig. 2*C*).

When additional fatty acids were not provided, expression of *SbDES3* in yeast yielded no new products. Only strains expressing *SbDES3* cultivated in the presence of exogenously supplied linoleic acid (18:2$\Delta^{9,12}$) produced an additional peak, with a GC retention time of 41.15 min, and accounted for ~2.1% of the total cellular fatty acids (Fig. 6*B* and Table 1). This peak was identified as α-linolenic acid (18:3$\Delta^{9,12,15}$), based on the comparison of the FAME mass spectrum (Fig. 6*E*) to that of authentic standards, as well as GC/MS analysis of the corresponding DMOX derivatives (Fig. 7*B*). Collectively, these results suggest that the preferred substrates for SbDES2 and SbDES3 differed, and were 16:1Δ^9 and 18:2$\Delta^{9,12}$ fatty acids, respectively, under these experimental conditions.

Co-expression of SbDES2 and SbDES3 in S. cerevisiae Results in the Production of a 16:3 Fatty Acid Containing a Terminal Double Bond—As discussed above, expression of *SbDES2* in yeast resulted in the production of 16:2$\Delta^{9,12}$ fatty acid, a proposed precursor to the 16:3$\Delta^{9,12,15}$ starter unit used by PKSs associated with the sorgoleone biosynthetic pathway (15, 16). Given the ability of SbDES3 to catalyze the formation of α-linolenic acid (18:3$\Delta^{9,12,15}$) from linoleic acid (18:2$\Delta^{9,12}$), we postulated that this 16:2 fatty acid would serve as a substrate for SbDES3. To test this hypothesis, yeast cells simultaneously expressing both fatty acid desaturases were engineered. Co-expression of *SbDES2* and *SbDES3* in yeast resulted in the appearance of a new peak with a retention time of 29.4 min (Fig. 6*C*) and parent ion mass of 264 (Fig. 6*F*), in addition to the 16:2 peak detected following expression of *SbDES2* alone (Fig. 6*A*). The ion fragments observed in the corresponding mass spectrum were consistent with the fragmentation of a 16:3 fatty acid possessing a terminal double bond (Fig. 6*F* and compare with Fig. 2*D*). Moreover, the mass spectrum for the DMOX derivative (Fig. 7*C*) was identical to recently reported mass spectral data for 16:3$\Delta^{9,12,15}$ (42). Similar results were obtained when exogenous palmitic (16:0), palmitoleic (16:1Δ^9), oleic (18:1Δ^9), α-linolenic (18:3$\Delta^{9,12,15}$), or γ-linolenic acid (18:3$\Delta^{6,9,12}$) were supplemented to the media, and as expected, a peak corresponding to 18:3$\Delta^{9,12,15}$ (Fig. 6, *B* and *E*) was also observed in addition to the 16:2 and 16:3 peaks when linoleic acid (18:2$\Delta^{9,12}$) was provided (data not shown).

To further confirm the identity of the putative 16:3$\Delta^{9,12,15}$ fatty acid, the corresponding FAME was isolated by preparative TLC, and NMR spectroscopy was performed. The resulting GC/MS profile of the product isolated by TLC was consistent with that of the FAME peak observed in the initial experiments (Fig. 6*C*). The ^{1}H NMR chemical shifts of this isolate confirmed the presence of terminal methylene protons (δ 4.98, br d, J = 17 Hz, 1H, H-16a; 5.02 br d, J = 10 Hz, 1H, H-16b) and the three double bonds (δ 5.80, m, 1H, H-15; δ 5.30, m, 4H, H-9, 10, 12, 13) separated by methylene groups (δ 2.78, m, 4H, H-11, 14) (6, 10). Importantly, the results of these analyses reveal that sorghum SbDES3 catalyzes the conversion of 16:2$\Delta^{9,12}$ to the unusual 16:3$\Delta^{9,12,15}$ fatty acid, which possesses a terminal double bond.

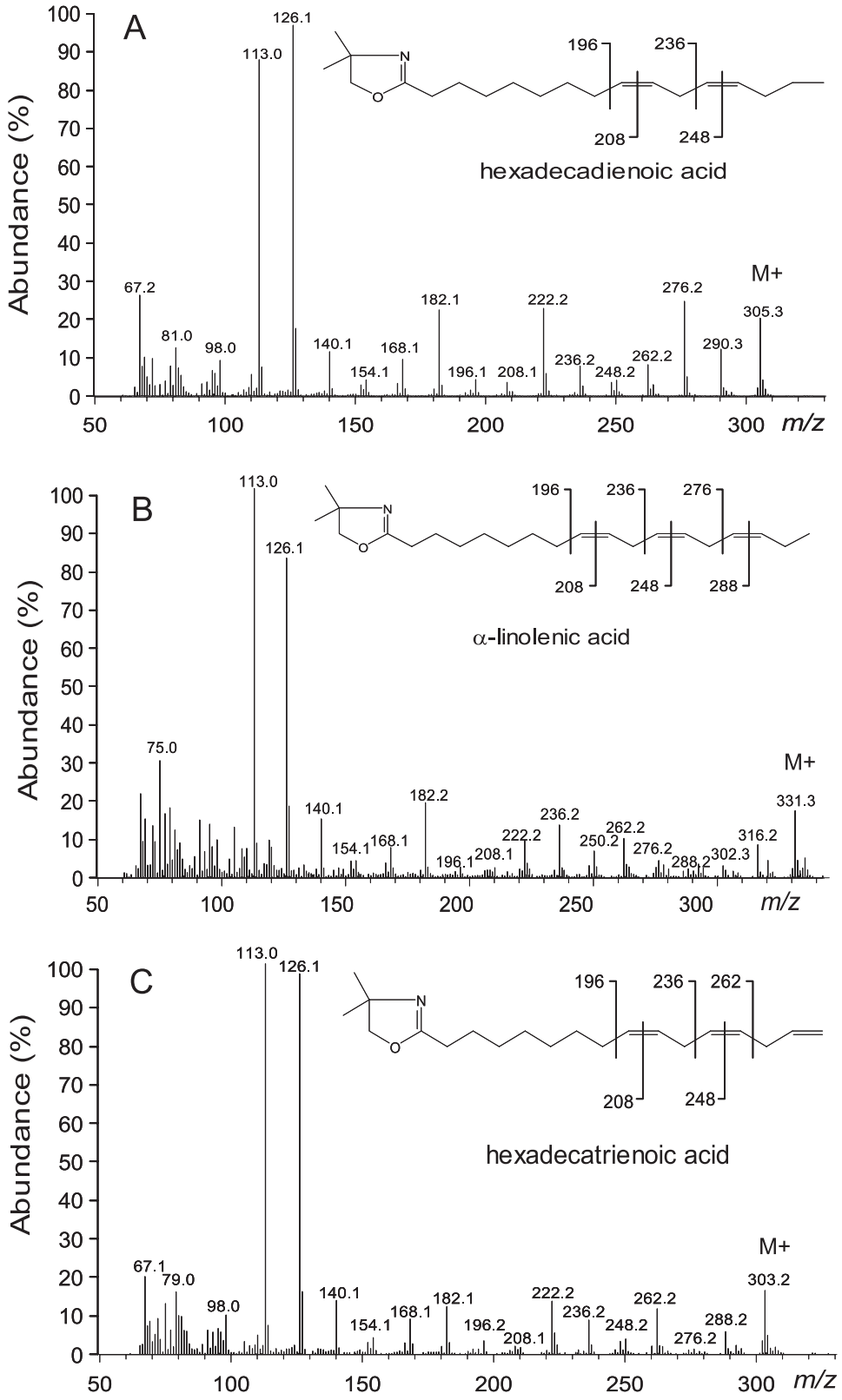

FIGURE 7. **Mass spectra of 16:2, 18:3, and 16:3 DMOX derivatives prepared from yeast cells expressing *S. bicolor* desaturases.** *A*, 16:2 DMOX derivative from cells expressing *SbDES2*; *B*, 18:3 DMOX derivative from cells expressing *SbDES3*, *C*, mass spectrum of 16:3 DMOX derivative from cells co-expressing *SbDES2* and *SbDES3*.

DISCUSSION

A unique feature of the chemical structure of the allelochemical sorgoleone is the presence of a terminal double bond in the aliphatic side chain, which has generated significant interest in the investigation of the sorgoleone biosynthetic pathway. In the present work, we have identified two fatty acid desaturases from sorghum (*S. bicolor* genotype BT×623) that catalyze consecutive desaturation steps on a 16:1 fatty acid precursor: a Δ^{12} desaturase (SbDES2) that converts *cis*-palmitoleic acid (16:1Δ^{9}) to hexadecadienoic acid (16:2$\Delta^{9,12}$), and a Δ^{15} desaturase (SbDES3) that is capable of catalyzing the formation of a terminal double bond on a 16:2$\Delta^{9,12}$ fatty acid, yielding the 16:3$\Delta^{9,12,15}$ precursor from which the aliphatic side chain of sorgoleone originates (15, 16).

Of the three fatty acid desaturases preferentially expressed in root hair cells, both SbDES2 and SbDES3 exhibited activity in the heterologous yeast expression system used in the present work. SbDES1 exhibited no detectable activity, although it shares 89.7% identity and 92% similarity with SbDES3 at the amino acid level, and was also positioned within the same clade (corresponding to Δ^{15}-desaturases) by phylogenetic analysis (Fig. 5). The reason for this apparent lack of SbDES1 activity in transformed yeast is at present not clear, however, the functionality could potentially be improved through the use of an alternative host system, as previously observed in the case of CvFAD3 from *C. vulgaris* (43). Initial expression of *CvFAD3* in yeast did not yield the expected product linolenic acid in these studies; however, activity was subsequently obtained by expressing the cDNA in transgenic tobacco plants. The authors postulated that the lack of activity using the yeast system could potentially be because of translational repression, or alternatively, aberrant targeting to a cellular compartment where the catalytic activity was hindered or the appropriate substrates were unavailable. Although in the present work desaturation of the terminal carbon-carbon bond of 16:2$\Delta^{9,12}$ fatty acid by SbDES3 was achieved via co-expression with SbDES2 in yeast cells, because of the low Δ^{15}-desaturation

activity on linoleic acid (18:2$\Delta^{9,12}$) (Fig. 6*B*) and the potentially limited availability and/or accessibility of the hexadecadeinoic acid (16:2$\Delta^{9,12}$) substrate *in vivo*, it is difficult to infer the relative activity of SbDES3 for omega-3 *versus* terminal desaturation from the present data. The preference of the enzyme for 16:2 *versus* 18:2 substrates *in planta* is also unclear at the present time. The observation of relatively high levels of 18:3 fatty acids in all sorghum tissues analyzed (Fig. 2), combined with the real-time RT-PCR results indicating that *SbDES3* transcripts accumulate predominantly in root hairs (Fig. 3), suggests that FAD3-type enzymes other than *SbDES3* are responsible for the majority of linoleic acid (18:3) biosynthesis occurring in sorghum. However, as SbDES3 protein levels were not determined in the present work, the possibility cannot be excluded that sufficient SbDES3 activity exists in tissues other than root hairs which could account, at least in part, for the observed linoleic acid levels (Fig. 2).

Currently available evidence indicates that the biosynthesis of sorgoleone initiates with the formation of an unusual 16:3$\Delta^{9,12,15}$ fatty acid, which serves as the starter molecule for a polyketide synthase activity which yields an alkylresorcinol intermediate possessing a 15-carbon aliphatic side chain. The identification of an abundant pool of 16:3 free fatty acids specifically in sorghum root hairs is consistent with the hypothesis that the 16:3 fatty acid serves as the biosynthetic precursor, and that sorgoleone biosynthesis is restricted to this cell type. This notion is further supported by the recent isolation of cDNAs from *S. bicolor* (genotype BT×623) encoding polyketide synthases capable of catalyzing the formation of 5-pentadecatrienyl resorcinol from 16:3 fatty acid and malonyl-CoA *in vitro*, which are also preferentially expressed in root hair cells at the steady-state mRNA level.[7]

In the majority of plant species analyzed, substantial amounts of 16:3 fatty acids esterified to galactolipids accumulate in leaves, generally possessing a $\Delta^{7,10,13}$ configuration (44, 45). It would therefore be of considerable interest to investigate the effects of fatty acids possessing terminal methylene groups within a biological context, given that polyunsaturated fatty acids not only are essential components of cellular membranes, but also influence physiological processes critical to plant survival such as tolerance to cold temperature extremes (46). Expression of an *Arabidopsis* ω-3 fatty acid desaturase gene (*Fad7*) in transgenic tobacco, for example, increased the level of trienoic acids (16:3 and 18:3) considerably and led to a dramatic enhancement in cold tolerance, associated with an increase in photosynthetic capacity at low temperatures. Experiments designed to study the physiological effects of producing a fatty acid with a terminal double bond in transgenic *Arabidopsis* and *S. bicolor* plants via overexpression of *SbDES2* and *SbDES3* are currently underway in our laboratory.

An important caveat concerning assigning functions to novel desaturases based on heterologous expression data, is that desaturase functionality can be significantly affected by the metabolic context of the host cells (47). Additionally, the presence of highly active acyltransferases in yeast render the determination of the precise form of the substrate utilized *in vivo* (acyl-CoA *versus* lipid-linked) less than straightforward (discussed in Domergue *et al.*, 26). Nevertheless, the demonstration in the current work that: 1) co-expressed *SbDES2* and *SbDES3* sequentially catalyze the conversion of 16:1Δ^{9} fatty acid to the atypical 16:3$\Delta^{9,12,15}$ fatty acid in yeast, 2) 16:3$\Delta^{9,12,15}$ is almost exclusively found in sorghum root hairs where *SbDES2* and *SbDES3* are highly and predominantly expressed (Figs. 2 and 3), combined with, 3) previous studies demonstrating the likelihood that 16:3$\Delta^{9,12,15}$ CoA ester serves as the precursor for the allelopathic benzoquinone sorgoleone (15, 16) for which biosynthesis occurs predominantly or exclusively in root hair cells (13, 14), collectively provide compelling evidence that SbDES2 and SbDES3 represent key enzymes in sorgoleone biosynthesis. Gene knock-out approaches with transgenic sorghum plants, which will be pursued in our future studies, could provide unequivocal evidence for their involvement in this pathway.

The identification and functional characterization of SbDES3 extends the range of outcomes that have been ascribed to the FAD3 family of enzymes, to which can now be included the capacity to desaturate the terminal carbon-carbon bond in a 16:2$\Delta^{9,12}$ fatty acid. Furthermore, the characterization of both SbDES2 and SbDES3 provides promising new tools for elucidating the regulatory mechanisms governing allelochemical interactions in plants, for which there is currently a paucity of information. Efforts by our laboratory to identify genes encoding the remaining enzymes required for sorgoleone biosynthesis are ongoing, to facilitate metabolic engineering experiments in transgenic *S. bicolor* plants targeting this pathway.

Acknowledgments—We thank Marilyn Ruscoe, Melanie Mask, Gloria Hervey, and Mark Holtan for excellent technical assistance. We also thank Dr. John Shanklin for critically reviewing this manuscript and for numerous helpful discussions.

[7] D. Cook, F. E. Dayan, A. M. Rimando, N. P. D. Nanayakkara, Z. Pan, J. Schröder, M. Fishbein, S. O. Duke, and S. R. Baerson, manuscript in preparation.

REFERENCES

1. Duke, S. O., Rimando, A. M., Baerson, S. R., Scheffler, B. E., Ota, E., and Belz, R. G. (2002) *J. Pest. Sci.* **27,** 298–306
2. Inderjit, and Duke, S. O. (2003) *Planta* **217,** 529–539
3. Bais, H. P., Park, S. W., Weir, T. L., Callaway, R. M., and Vivanco, J. M. (2004) *Trends Plant Sci.* **9,** 26–32
4. Einhellig, F. A. (1996) *Agron. J.* **88,** 886–893
5. Netzly, D. H., and Butler, L. G. (1986) *Crop Sci.* **26,** 775–778
6. Chang, M., Netzly, D. H., Butler, L. G., and Lynn, D. G. (1986) *J. Am. Chem. Soc.* **108,** 7858–7860
7. Einhellig, F. A., Rasmussen, J. A., Hejl, A. M., and Souza, I. F. (1993) *J. Chem. Ecol.* **19,** 369–375
8. Rasmussen, J. A., Hejl, A. M., Einhellig, F. A., and Thomas, J. A. (1992) *J. Chem. Ecol.* **18,** 197–207
9. Gonzalez, V. M., Kazimir, J., Nimbal, C. I., Leslie A. Weston, and Cheniae, G. M. (1997) *J. Agric. Food Chem.* **45,** 1415–1421
10. Rimando, A. M., Dayan, F. E., Czarnota, M. A., Weston, L. A., and Duke, S. O. (1998) *J. Nat. Prod.* **61,** 927–930
11. Czarnota, M. A., Paul, R. N., Dayan, F. E., Nimbal, C. I., and Weston, L. A. (2001) *Weed Technol.* **15,** 813–825
12. Duke, S. O. (2003) *Trends Biotechnol.* **21,** 192–195
13. Czarnota, M. A., Paul, R. N., Weston, L. A., and Duke, S. O. (2003) *Int. J. Plant Sci.* **164,** 861–866
14. Czarnota, M. A., Rimando, A. M., and Weston, L. A. (2003) *J. Chem. Ecol.* **29,** 2073–2083

15. Fate, G. D., and Lynn, D. G. (1996) *J. Am. Chem. Soc.* **118,** 11369–11376
16. Dayan, F. E., Kagan, I. A., and Rimando, A. M. (2003) *J. Biol. Chem.* **278,** 28607–28611
17. Netzly, D. H., Riopel, J. L., Ejeta, G., and Butler, L. G. (1988) *Weed Sci.* **36,** 441–446
18. Einhellig, F. A., and Souza, I. F. (1992) *J. Chem. Ecol.* **18,** 1–11
19. Behrouzian, B., and Buist, P. H. (2002) *Curr. Opin. Chem. Biol.* **6,** 577–582
20. Martin, C. E., Oh, C. S., Kandasamy, P., Chellapa, R., and Vemula, M. (2002) *Biochem. Soc. Trans.* **30,** 1080–1082
21. Wallis, J. G., Watts, J. L., and Browse, J. (2002) *Trends Biochem. Sci.* **27,** 467
22. Bucher, M., Schroeer, B., Willmitzer, L., and Riesmeier, J. W. (1997) *Plant Mol. Biol.* **35,** 497–508
23. Baerson, S. R., Sanchez-Moreiras, A., Pedrol-Bonjoch, N., Schulz, M., Kagan, I. A., Agarwal, A. K., Reigosa, M. J., and Duke, S. O. (2005) *J. Biol. Chem.* **280,** 21867–21881
24. Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. (1997) *Nucleic Acids Res.* **25,** 4876–4882
25. Saitou, N., and Nei, M. (1987) *Mol. Biol. Evol.* **4,** 406–425
26. Domergue, F., Abbadi, A., Zahringer, U., Moreau, H., and Heinz, E. (2005) *Biochem. J.* **389,** 483–490
27. Felsenstein, J. (1985) *Evolution* **39,** 783–791
28. Burke, D., Dawson, D., and Stearns, T. (2000) *Methods in Yeast Genetics*, pp. 103–105, Cold Spring Harbor Laboratory Press, NY
29. Christianson, T. W., Sikorski, R. S., Dante, M., Shero, J. H., and Hieter, P. (1992) *Gene* (*Amst.*) **110,** 119–122
30. Dyer, J. M., Chapital, D. C., Kuan, J.-C. W., Mullen, R. T., Turner, C., McKeon, T. A., and Pepperman, A. B. (2002) *Plant Physiol.* **130,** 2027–2038
31. Peyou-Ndi, M. M., Watts, J. L., and Browse, J. (2000) *Arch. Biochem. Biophys.* **376,** 399–408
32. Fay, L., and Richli, U. (1991) *J. Chromatogr.* **541,** 89–98
33. Cordonnier-Pratt, M.-M., Liang, C., Wang, H., Kolychev, D. S., Sun, F., Freeman, R., Sullivan, R., and Pratt, L. H. (2004) *Comp. Funct. Genom.* **5,** 268–275
34. Los, D. A., and Murata, N. (1998) *Biochim. Biophys. Acta* **1394,** 3–15
35. Shanklin, J., and Cahoon, E. B. (1998) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **49,** 611–641
36. Yang, X., Scheffler, B. E., and Weston, L. A. (2004) *J. Exp. Bot.* **55,** 2251–2259
37. Shanklin, J., Whittle, E., and Fox, B. G. (1994) *Biochemistry* **33,** 12787–12794
38. Browse, J., McConn, M., James, D., Jr., and Miquel, M. (1993) *J. Biol. Chem.* **268,** 16345–16351
39. Kodama, H., Akagi, H., Kusumi, K., Fujimura, T., and Iba, K. (1997) *Plant Mol. Biol.* **33,** 493–502
40. Horiguchi, G., Fuse, T., Kawakami, N., Kodama, H., and Iba, K. (2000) *Plant J.* **24,** 805–813
41. Stukey, J. E., McDonough, V. M., and Martin, C. E. (1989) *J. Biol. Chem.* **264,** 16537–16544
42. Sayanova, O., Haslam, R., Guschina, I., Lloyd, D., Christie, W. W., Harwood, J. L., and Napier, J. A. (2006) *J. Biol. Chem.* **281,** 36533–36541
43. Suga, K., Honjoh, K., Furuya, N., Shimizu, H., Nishi, K., Shinohara, F., Hirabaru, Y., Maruyama, I., Miyamoto, T., Hatano, S., and Iio, M. (2002) *Biosci. Biotechnol. Biochem.* **66,** 1314–1327
44. Browse, J., and Somerville, C. R. (1991) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **42,** 467–506
45. Mongrand, S., Bessoule, J.-J., Cabantous, F., and Cassagne, C. (1998) *Phytochemistry* **49,** 1049–1064
46. Miquel, M., James, D., Jr., Dooner, H., and Browse, J. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 6208–6212
47. Heilmann, I., Pidkowich, M. S., Girke, T., and Shanklin, J. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 10266–10271